Multiplex Analysis of Heterophil Antibodies in Patients With Indeterminate HIV Immunoassay Results

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Abstract

We hypothesized that heterophil antibodies reactive with animal proteins used in blot preparation caused nonspecific staining (NSS) on HIV Western blot (WB) studies, causing indeterminate results. We analyzed samples showing NSS on HIV WB using a multiplexed immunoassay to simultaneously measure IgG antibodies to animal IgG (bovine, goat, sheep, mouse) and bovine serum albumin. Heterophil antibodies reactive with IgG from several animal species were detected in 23 (49%) of 47 samples showing NSS on HIV WB; 15 positive samples demonstrated antibodies to all 5 antigens. Similar IgG heterophil antibodies were detected in only 2 (8%) of 24 control samples. Of the HIV WB samples with a positive HIV-1 enzyme-linked immunosorbent assay (ELISA) result at the time of WB testing (11/47), heterophil antibodies were found in 8 (73%) of 11. Preabsorption with bovine, goat, and sheep IgG removed heterophil antibodies detected by the multiplexed assay and, in some cases, eliminated reactivity in ELISA and WB testing. Heterophil antibodies are associated with indeterminate HIV immunoassay results and are an important cause of false-positive HIV ELISA results. Multiplexed immunoassays provide a powerful tool for screening patients for heterophil antibodies and resolving possible false-positive results.

Heterophil antibodies are widely recognized as a cause of interference in immunoassays. Heterophil antibodies are thought to demonstrate multispecificity by having multiple binding sites or by having a single binding site that can recognize a number of antigens with similar structures.[1] Heterophil antibodies can create a false-positive result in an immunoassay by binding to the capture and signal antibodies in the assay and mimicking the behavior of the analyte of interest. Heterophil interference has been observed in many immunoassays, including creatine kinase MB,[2] human chorionic gonadotropin,[3] CA-125,[4] thyroid stimulating hormone,[5] and others, but has not been well characterized in HIV immunoassays.[6]

False-positive HIV enzyme-linked immunosorbent assay (ELISA) results can occur with serious medical, social, and legal consequences,[7] and false-positive results are thought to account for as many as 4.6% of positive Western blots in a low-risk screening setting.[8] The HIV Western blot can yield indeterminate results because of nonviral bands or cross-reacting autoantibodies.[9,10] Nonspecific staining (NSS) is not addressed specifically in the criteria for interpreting HIV Western blot assays.[11] Since NSS can obscure a viral band on the blot, many laboratories will not interpret a blot with NSS as negative but instead report a result of indeterminate with a recommendation for retesting. We hypothesized that the majority of NSS observed on HIV Western blots was due to heterophil antibodies, since animal proteins, including immunoglobulins, are used in the manufacture of the blots. Furthermore, any sample with heterophil antibodies that caused NSS on a Western blot also might show a false-positive result on an ELISA.

To detect heterophil antibodies in samples showing NSS that were potentially reactive with many different antigens,

we used microsphere-based multiplexed immunoassays on the Luminex platform (Luminex, Austin, TX). The Luminex analyzer performs flow cytometric analysis of microsphere-based immunoassays with simultaneous measurement of multiple analytes.[12] We used a panel of animal serum proteins commonly used in immunoassays, including polyclonal bovine, goat, sheep, and mouse IgG and bovine serum albumin (BSA). In the present study, we screened for a subset of heterophil antibodies likely to cause interference in immunoassays by simultaneously measuring human IgG reactive with these antigens. We report that human serum samples producing NSS on HIV Western blots often contain heterophil IgG antibodies reacting with a variety of serum proteins from other animal phyla.

Materials and Methods

Patients and Serum Samples

During a 5-month period (July 1, 1999-November 30, 1999), 2,518 consecutive samples were submitted to ARUP Laboratories, Salt Lake City, UT, for HIV Western blot testing. Samples were tested according to the manufacturer's protocol (Sanofi Diagnostics Pasteur, Redmond, WA). According to the manufacturer's recommendations, "Strips which are obscured by the development of dark blotches or marks should not be interpreted."[13] All blots showing NSS, as determined by the technologist at the time of testing, were given an interpretation of indeterminate for HIV-1 infection in the final laboratory report. Serum from any blot that showed NSS was selected for heterophil antibody analysis. All samples were tested simultaneously with an HIV-1 ELISA assay (Organon Teknika, Durham, NC). Serum was stored at –20°C. When available, patient information was obtained from the submitting physician.

Control Group

The control group was 24 serum samples from random blood donors. None of these samples were positive for hepatitis B core antibodies, hepatitis B surface antigen, hepatitis C antibodies, antibodies to human lymphotropic virus I or II, antibodies to HIV-1, or p24 antigen.

Multiplex Heterophil Immunoassay

Microsphere Preparation

BSA and bovine, goat, sheep, mouse, and human IgG (Sigma Chemical, St Louis, MO) were conjugated to 6 unique fluorescently labeled microsphere sets (Luminex) with a carbodiimide coupling method. Two hundred microliters of 1.25×10^{11} microspheres per liter stock solution (2.5×10^6 microspheres) was activated in 100 µL of a 0.1-mol/L concentration of sodium phosphate buffer containing 500 µg of *N*-hydroxysulfosuccinimide sodium. Microspheres were washed twice with 250 µL of phosphate-buffered saline (PBS) (pH 7.3), with centrifugation at 10,000*g* for 60 seconds, to harvest the microspheres. Activated, washed microspheres were suspended in 250 µL of a 50 µg/mL solution of BSA or animal IgG in PBS. After 1 hour, coated microspheres were washed twice with 250 µL of PBS with 0.2 mL/L of polysorbate 20, pH 7.3 (PBS/polysorbate), suspended in 1 mL of PBS/polysorbate, and enumerated on a hemocytometer. The final concentrations of the individual microspheres ranged from 1.1 to 1.7×10^3/mL. A solution of all 6 unique microspheres in PBS/polysorbate was made, with each microsphere at a concentration of 1×10^5 microspheres per milliliter.

Multiplexed Assay for Heterophil Antibodies

For this assay, 50 µL of a 1:50 dilution of patient serum in PBS and 50 µL of the 6-microsphere solution were mixed in a filter-bottomed microtiter well and incubated for 20 minutes. The microspheres were washed 3 times with 250 µL of PBS/polysorbate. Then, 100 µL of a 0.04-µg/mL solution of biotinylated goat antihuman IgG (Pierce Chemicals, Rockford, IL) was added to the microspheres and incubated for 20 minutes, and the microspheres were washed 3 times with 250 µL of PBS/polysorbate. Next, 100 µL of a 10-µg/mL solution of streptavidin-phycoerythrin conjugate (Molecular Probes, Eugene, OR) was added to the microspheres and incubated for 10 minutes, and the microspheres were washed 3 times with 250 µL of PBS/polysorbate. The microspheres were resuspended in 200 µL of PBS/polysorbate and assayed with the Luminex system.

Preabsorption of Heterophil Antibodies

For preabsorption, 5 mg/mL solutions of bovine, goat, and sheep IgG in PBS were used for binding to the coupling gel (product number 20501, Pierce Chemicals) according to the manufacturer's instructions. The gel with bound animal IgG was stored as a 50% slurry in PBS/polysorbate; 100 µL of each of the 3 gel slurries was added to 5 µL of patient serum and mixed. The gel mixture was incubated at room temperature for 60 minutes followed by centrifugation at 10,000*g* for 3 minutes. The supernatant was diluted to 1:50 in PBS and submitted for multiplex analysis. For preabsorption in the HIV-1 ELISA and Western blot (Sanofi Diagnostics Pasteur) assays, the supernatant was diluted in the appropriate specimen diluent and analyzed in the usual manner.

Results

From July 1, 1999, to November 30, 1999, 2,518 HIV Western blots were performed. Of these, 47 (1.9%) showed

NSS. Examples of blots showing NSS are shown in **Image 1**. NSS varied from strong, dark, diffuse staining to light, blotchy staining. However, any staining that potentially could obscure the identification of a viral band was recorded as NSS, and the result was interpreted as indeterminate for HIV infection.

Patient information was available for 25 of the 47 samples showing NSS. One patient was a homosexual male, but no other patients had known risk factors for HIV infection. In many cases, testing was performed for routine prenatal care, blood donation, or evaluation for dialysis or transplantation or because a health care worker was exposed to the patient's blood. Six months after the time of Western blot testing, none of the patients for whom information was available were known to be HIV positive.

Of the 47 samples, 11 (23%) had a positive HIV-1 ELISA result, and results for 36 (77%) were negative. Presumably all of these patients had a positive HIV ELISA result in another laboratory, since samples were sent to our laboratory for confirmatory Western blot testing only. Each of the 11 samples with a positive HIV-1 ELISA result in our laboratory was only slightly above the positive cutoff for the assay. Most true-positive samples have signal/cutoff ratios greater than 5.0, while the ELISA-positive samples in our study had signal/cutoff ratios ranging from 1.02 to 2.98.

Figure 1 shows the results of the multiplex analysis for heterophil antibodies in 2 human serum samples demonstrating NSS in the HIV Western blot. Reactivity with cow, goat, sheep, and mouse IgG and BSA is shown. Each vertical bar represents the average amount of fluorescence measured on the microspheres labeled with a given antigen, corresponding to the amount of IgG antibody present. A separate microsphere coated with human IgG used to demonstrate adequate labeling with the antihuman secondary antibody is not shown. The positive cutoff for each antigen was the mean reactivity of the blood bank population for that antigen plus 2.5 SD. The positive cutoff mean fluorescence intensities were as follows: cow IgG, 6,580; goat IgG, 7,450; sheep IgG, 7,770; mouse IgG, 1,830; and BSA, 2,150.

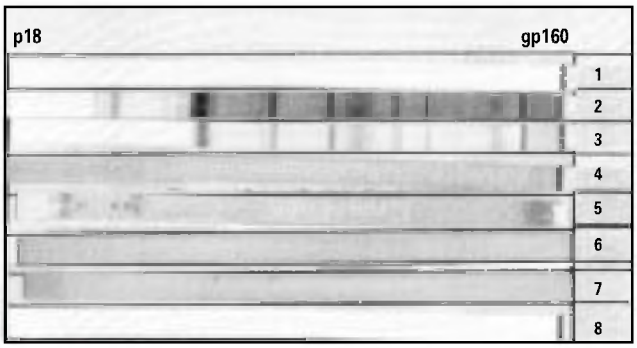

Image 1 Examples of nonspecific staining on HIV Western blots. Blots 1 and 8, negative controls; blots 2 and 3, positive controls; blots 4-7, nonspecific staining. Blots 4-7 show various levels of diffuse darkening in comparison with blots 1 and 8 (negative controls). gp, glycoprotein.

Multiplex analysis of control blood bank samples for heterophil antibodies indicated 2 (8%) of 24 had heterophil antibodies. These results are shown in **Figure 2**. In the samples that contained heterophil antibodies, one contained antibodies to all 5 heterophil antigens, while the other had antibodies to only mouse IgG.

As shown in Figure 2, multiplex analysis of the samples showing NSS on HIV Western blot indicated that 23 (49%) of 47 had heterophil antibodies directed against 1 or more animal species IgG. This is significantly higher than the results for the control population ($P < .001$). Fifteen samples contained antibodies reactive with all 5 antigens, 1 sample showed reactivity to 4 antigens, 3 samples showed reactivity to 3 antigens, 1 sample showed antibodies to 2 antigens, and 3 samples had antibodies to only 1 antigen. For each heterophil antigen, the results were as follows: 20 samples had antibodies to mouse IgG, 19 samples contained antibodies to bovine IgG, 19 samples were positive for antibodies to sheep IgG, 18 samples had antibodies to goat IgG, and 18 samples contained antibodies to BSA.

Of the 11 samples that were positive in our laboratory by HIV-1 ELISA, 8 (73%) contained heterophil antibodies. Of the 36 samples that were negative in our laboratory by HIV-1 ELISA, 14 (39%) had heterophil antibodies by our analysis. These rates are significantly different ($P < .05$).

We next attempted to remove the heterophil antibodies from the patient serum by preabsorbing the serum with cow, goat, or sheep IgG bound to a gel solid phase. Antibodies with these specificities were chosen for preabsorption because they were present in the highest titers in the multiplex analysis. Preabsorption was performed on a subset of samples that had shown a positive result on HIV-1 ELISA. Preabsorbed patient serum samples showed marked reduction in the levels of heterophil antibodies, as shown in **Table 1**. In almost all cases, after preabsorption with bovine, goat, and sheep IgG, heterophil antibody levels for all 5 heterophil antigens were below the positive cutoff. One sample continued to show antibodies to mouse IgG above the positive cutoff after preabsorption.

Preabsorbed serum samples also were retested using the HIV-1 ELISA and HIV Western blot. Positive and negative control samples also were preabsorbed and reacted appropriately following preabsorption. Of 9 samples initially positive by HIV-1 ELISA, 5 were negative after preabsorption and 4 remained positive. The samples that remained positive had shown antibodies to all 5 antigens; however, after absorption only 1 sample showed remaining heterophil antibodies that

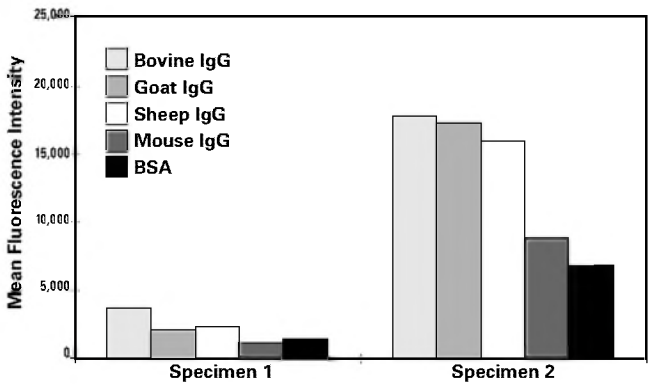

Figure 1 Two specimens analyzed for immunoreactivity to 5 antigens, represented by 5 vertical bars. Increasing mean fluorescence intensity represents increasing patient antibody reacting with a particular antigen. BSA, bovine serum albumin.

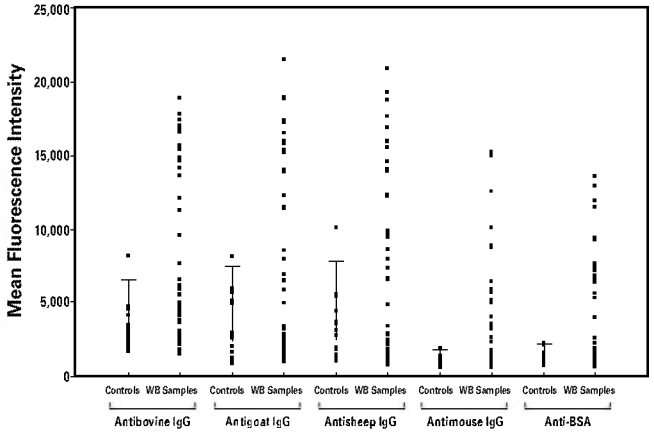

Figure 2 Multiplex analysis for heterophil antibodies in the blood bank control population and in samples showing nonspecific staining on HIV Western blot (WB). The error bars for the control population represent the mean + 2.5 SD. Of 47 samples, 23 (49%) with nonspecific staining showed significant levels of heterophil antibodies to 1 or more antigens, while only 2 (8%) of 24 of the control population had heterophil antibodies.

had specificity for only mouse IgG. Presumably, remaining interfering antibodies had different specificities.

Five samples were retested by HIV Western blot after preabsorption, and 3 of these were negative with no NSS. Two samples continued to show NSS. These samples did not show heterophil antibodies on multiplex analysis after preabsorption, so that any antibodies continuing to cause interference presumably had different specificities.

Discussion

Indeterminate and false-positive results in HIV immunoassays are a substantial problem for physicians, public health authorities, and, especially, patients; recognition of false-positive results potentially can alleviate much confusion and anxiety. Heterophil antibodies are common in the general population[14-16] and frequently cause interference and false-positive results in many types of immunoassays.[2-5,7,17-19] Heterophil antibodies have not previously been shown definitively to interfere in HIV immunoassays.[6] With a unique multiplexed immunoassay, we have demonstrated that heterophil antibodies can cause false-positive HIV ELISA results in cases in which there are no known risk factors and clinical suspicion is low. In many cases, heterophil antibodies cause additional problems in HIV testing because they interfere in the confirmatory Western blot test, resulting in NSS and an interpretation of an indeterminate result for HIV infection.

Table 1
Heterophil Antibody Content and HIV-1 ELISA and HIV WB Results Before and After Antibody Preabsorption

	Antibovine IgG*		Antigoat IgG*		Antisheep IgG*		HIV-1 ELISA Signal/Cutoff†		HIV-1 WB	
Sample	Before	After	Before	After	Before	After	Before	After	Before	After
1	16,806	2,262	13,926	837	14,636	1,861	2.33 (+)	1.74 (+)	—	—
2	4,479	1,303	5,012	294	7,371	836	2.13 (+)	0.35 (–)	NSS	Negative
3	17,433	3,554	18,895	795	18,771	2,097	1.74 (+)	1.51 (+)	NSS	NSS
4	6,180	1,741	7,972	435	9,681	2,941	1.03 (+)	0.44 (–)	NSS	Negative
5	18,949	2,608	21,532	1,079	20,888	1,793	2.98 (+)	1.48 (+)	NSS	NSS
6	14,704	2,829	18,960	1,403	12,389	3,178	1.45 (+)	0.78 (–)	NSS	Negative
7	17,845	2,349	17,277	834	15,960	1,895	1.58 (+)	0.60 (–)	—	—
8	14,924	1,973	15,405	482	16,933	1,521	1.05 (+)	1.43 (+)	—	—
9	9,569	2,078	5,853	462	6,617	1,177	1.02 (+)	0.48 (–)	—	—

ELISA, enzyme-linked immunosorbent assay; NSS, nonspecific staining; WB, Western blot.
* Measured as mean fluorescence intensity.
† A signal/cutoff ratio greater than 1.0 is a positive result.

We detected and characterized heterophil antibodies by multiplexed microsphere-based immunoassays, which allow simultaneous measurement of immunoreactivity to multiple antigens. Because heterophil antibodies characteristically react with immunoglobulins from multiple species,[20] multiplex analysis is ideal for their detection. In our analysis we used polyclonal bovine, goat, sheep, and mouse IgG and BSA, because these are common components of commercial immunoassays. Most positive samples (16/23 [70%]) in our analysis showed antibodies to at least 4 of these antigens. The specific animal proteins used in the commercial HIV immunoassay kits are not indicated by the manufacturer, although the specimen diluents are reported to contain bovine, caprine, and milk proteins[13,21]; presumably these are selected to match the proteins present on the solid phase of the assay.

Previous reports of heterophil antibodies have not commonly measured immunoreactivity to multiple species' immunoglobulins,[14,15] and whether reactivity to the combination of heterophil antigens we tested is characteristic is not clear. One possible explanation is that all of these species have immunoglobulins that are antigenically similar. This could explain why most patients showed antibodies to multiple species and preabsorption with only 1 antigen reduced the level of antibodies to all of the antigens measured. To further characterize heterophil antibodies in future studies, multiplex testing could examine immunoreactivity for many more antigens and also could include specific immunogens such as monoclonal mouse therapeutic agents or specific monoclonal antibodies used as radiolabeling agents. Such a panel could contain the immunoglobulins or proteins most frequently used in immunoassays and the monoclonal therapeutic agents most commonly used in humans.

If the use of multiplexed microsphere-based assays becomes more common and panels of tests are performed on this platform, a set of heterophil antigen microspheres could be added to the panel to monitor heterophil antibody activity in all samples tested. For example, if a panel included microspheres to detect antibodies to HIV, hepatitis B antigens, and hepatitis C, additional microspheres with a set of heterophil antigens could simultaneously detect heterophil antibodies and raise suspicion for interference in other immunoassays.

Once heterophil antibodies were identified in a patient with a suspected interference on HIV immunoassays, in some cases we were able to remove the antibodies by incubation with heterophil antigens. In our analysis, more than half of false-positive HIV-1 ELISA results and NSS on Western blot were removed by incubation with polyclonal bovine, goat, and sheep IgG to remove heterophil antibodies. Other investigators have also been able to eliminate heterophil antibody activity by preabsorption,[2,22,23] although some studies used animal serum while others used immunoglobulins only or F(ab') fragments.

In our study, preabsorption reduced antibody levels to those of the control population except for 1 sample that continued to show antibodies reactive with mouse IgG. The continued false-positive result of some samples after preabsorption was probably due to antibodies with other specificities that had not been removed.

Multiplex microsphere-based immunoassays are an excellent means for detecting heterophil antibodies. By using this technology, we have shown heterophil antibodies can be an important cause of false-positive HIV immunoassay results, and heterophil interference should be suspected in any case in which the HIV immunoassay results are at odds with the clinical situation. Additional studies to further characterize the specificity of heterophil antibodies, to detect heterophil interference in other settings, and to remove heterophil antibodies from clinical samples will be necessary to determine the scope of the problem and effective means of controlling it.

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